FT-IR spectroscopy, a reliable method for routine analysis of the degree of methylesterification of pectin in different fruit- and vegetable-based matrices

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Abstract

The use of Fourier transform infrared (FT-IR) spectroscopy as a method for routine analysis of the degree of methylesterification (DM) of pectin was validated. The relationship between the ratio of the intensity of the peak at around 1740 cm^{-1} (due to ester carbonyl group stretching) to the sum of the intensities of the peaks at around 1740 and 1630-1600 cm^{-1} (due to carboxylate group stretching) and the DM of pectin in model and real systems was investigated. In model systems of low to medium DM with low added protein (≤ 20%), accurate DM determinations were obtained without spectra deconvolution while for medium to high DM pectin with high added protein (≥ 30%), peak deconvolution was vital. In real systems, good DM determinations were obtained without peak deconvolution except for broccoli-derived samples. Considering that broccoli is a protein-rich vegetable, better determinations of the DM were obtained using deconvoluted FT-IR spectra.

Keywords: Fruits and vegetables; Pectin; Degree of methylesterification; FT-IR spectroscopy; Spectra deconvolution

1. Introduction

Plant cell walls are principally composed of polysaccharides in addition to minor compositions of proteins, lignin and various inorganic compounds. In fruits and vegetables, where the edible portions generally consist of parenchyma cells and intercellular spaces, cell walls are largely constituted of primary cell walls, in which pectin is the most abundant polysaccharide (Van Buggenhout, Sila, Duvetter, Van Loey, & Hendrickx, 2009; Van Buggenhout et al., 2009). Pectin *in situ* defines the mechanical and functional properties of the cell wall, during ripening of fruits over storage to processing of fruit- and vegetable-based products. Extracted pectin on the other hand is a commercially important polysaccharide in the food processing industry, where it is widely used as a food additive with gelling and stabilizing properties in jams, jellies, marmalades, milk and confectionery products (Sakai, Sakamoto, Hallaert, & Vandamme, 1993). The functionality of pectin is also important in other industries including the pharmaceutical industry and waste treatment (Sila et al., 2009; Thakur, Singh, & Handa, 1997). Pectin is a complex heteropolysaccharide predominantly containing galacturonic acid (GalA) residues, in which varying proportions of the acid groups are present as methyl esters (Voragen, Coenen, Verhoef, & Schols, 2009). One of the most important factors that define the functional properties of pectin is the degree of methylesterification (DM). According to Voragen et al., (1995), DM is defined as the percentage carboxyl groups esterified with methanol. This pectin property varies widely among tissues, species (Sene, Mccann, Wilson, & Grinter, 1994) and throughout the life cycle of plants. During plant development, a reduction in DM is probably the most common feature, but is particularly notable in fruit ripening, where it decreases with progressive ripening, making pectin susceptible to degradation by hydrolases such as polygalacturonase leading to subsequent softening of the cell wall structure (Brummell, 2006). The presence of methyl ester

groups also affects cross-linking of pectinate molecules by Ca^{2+}, which plays a vital role in the organisation of primary cell wall polymers and may in turn influence the textural properties of fruits and vegetables (Barros et al., 2002). In addition, pectin can be modified to different extents by enzymatic and/or non-enzymatic reactions during food processing, leading to changes in its functionality. Owing to differences in sensitivity of the pectin-modifying enzymes to physical and chemical processing conditions, they can be manipulated through selective processing to achieve the desired structural/rheological properties (Van Buggenhout et al., 2009). For instance, the DM of pectin can be controlled through application of thermal and/or high-pressure processing to selectively (in)activate pectin methyl esterase (PME), an enzyme responsible for pectin demethylesterification. On the other hand, during thermal treatment, demethylesterification of pectin may occur by chemical reaction. Based on the DM, pectin can be subdivided into two classes: low DM pectins (DM < 50%) and high DM pectins (DM > 50%) (Chong, Simsek, & Reuhs, 2009). When extracted pectin is used in gelling applications, the DM dictates the conditions under which gels are formed and the gelling mechanism involved. High DM pectin requires high soluble solids (> 55%) and low pH (< 3.5) to gel while (amidated) low DM pectin requires divalent cations such as calcium ions and dissociated carboxylate ions (Thibault & Ralet, 2003). Concerning pectin used as stabiliser in acidified milk systems such as sour milk products and mixtures of fruit juices and milk, high DM pectin is important in minimising protein interactions that can occur during acidification, thereby reducing protein coagulation. This property is attributed to the remaining carboxylic functions that are sufficiently dissociated at low pH of acidified milk products (pH 3.2-4.5). Pectin will then electrostatically stick to the positive areas of the casein particles, producing a highly hydrated layer to the surface of the casein, which prevents aggregation (Thibault & Ralet, 2003).

Owing to the importance of the DM of pectin *in situ* in fruits and vegetables, as well as in gelling, stabilisation, thickening and other applications, several methods have been developed for its quantification. The most commonly used methods are based on colorimetry and chromatography (GC, GLC and HPLC). Galacturonic acid and methanol amounts are independently quantified after sample hydrolysis and the ratio of their molar amounts is used to calculate the DM (Manrique & Lajolo, 2002; Manrique & Lajolo, 2002; Barros et al., 2002). Other methods used for analysis of DM of pectin include qualitative analysis of DM through binding with monoclonal anti-HG antibodies (JIM5, JIM7, LM7, LM18, LM19, LM20 PAM1) (Willats, Knox, & Mikkelsen, 2006) as well as capillary electrophoresis (Zhong, Williams, Goodall, & Hansen, 1998), which gives a view on the intermolecular distribution of the DM. However, due to the disadvantages associated with these methods, mainly the complexity of the procedures, alternative methods have been proposed. Nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) and Fourier transform infrared (FT-IR) spectroscopy (Chatjigakis et al., 1998), which are more trouble-free instrumental techniques to determine the DM of pectin, have been implemented. These methods directly determine both the amount of ionized carboxylate and of ester carbonyl groups without hydrolysing the ester linkage (Chatjigakis et al., 1998).

The FT-IR method is simple, non-destructive, fast and exhibits high sensitivity. Based on the absorption intensity of different functional groups at different wave numbers in the near infrared region (1800-1500 cm^{-1}), particularly carboxylate (COO^{-}) and ester carbonyl (C=O) groups, the DM of a pectin-containing material can be determined (Szymanska-Chargot & Zdunek, 2013; Szymanska-Chargot & Zdunek, 2013; Barros et al., 2002). The objective of this paper is to validate FT-IR as a method for the routine analysis of the DM of pectin in a range of fruits and vegetables using a single standard calibration curve. Fruits and vegetables at different stages

ranging from freshly harvested, to stored and processed products were evaluated. The validation was achieved by comparing the DM values obtained using FT-IR spectroscopy to the DM values obtained by colorimetric analysis. The FT-IR calibration curve was obtained using various mixtures of polygalacturonic acid and commercial pectin with high DM as well as series of pectins with varying DMs created by enzymatic or chemical demethylesterification. Peak deconvolution (peak splitting), a concept initially addressed by Chatjigakis et al., (1998), was incorporated into the analysis of the FT-IR spectra to correct for any interferences due to the presence of proteins in the samples.

2. Materials and methods

2.1 Preparation of calibration samples (model systems)

Two sets of calibration samples were prepared. A series of polygalacturonic acid and commercial citrus pectin mixtures (see section 2.1.1) and a series of pectin samples demethylesterified either chemically or enzymatically to create pectin samples with different nanostructural characteristics including different DMs and patterns of methylesterification (see section 2.1.2).

2.1.1. Mixtures of polygalacturonic acid and commercial citrus pectin

Calibration samples of different DMs were obtained by mixing commercial citrus pectin (DM ~ 94%, Sigma-Aldrich, Belgium) with polygalacturonic acid (Sigma-Aldrich, Germany) in different ratios. A series of samples with DM 0 to 94% were obtained.

2.1.2. Chemically and enzymatically demethylesterified pectin samples

Commercial citrus pectin was either chemically or enzymatically demethylesterified according to the method of Ngouemazong et al. (2011). High DM citrus pectin (DM ~ 94%) was chemically demethylesterified to different DMs using NaOH. Pectin (0.8% w/w) was dissolved in demineralised water and adjusted to pH 11 with NaOH. Saponification of methylesters occurred at this pH, leading to a drop in pH, that was counteracted by titration with NaOH (0.1 M) using an automatic pH-stat titrator. To achieve the desired DM, a theoretically predetermined volume of NaOH was added to the pectin solution and the reaction was stopped by lowering the pH to 4.5 using HCl. Considering that chemical demethylesterification of pectin is a random process that results in a statistical distribution of free and methylesterified galacturonic residues (Limberg et al., 2000), the chemically demethylesterified pectin samples exhibited different DMs with random patterns of methylesterification.

Next, plant PME was extracted from carrots (*Daucus carota* cultivar Nantes) and purified as described in the method according to Jolie et al., (2009). Purified plant PME (15 U) was added to 25 mL of pectin solution (0.8% w/w high DM citrus pectin, DM ~ 94%) and incubated for predetermined times in a water bath at 30 °C. The samples were cooled in an ice bath and the pH was adjusted to 4.5 in order to reduce susceptibility of pectin to β-elimination during subsequent treatments. Thereafter, PME was inactivated by a 4 min heat treatment at 85 °C and the samples were rapidly cooled in an ice bath. As reported by Ngouemazong et al. (2011), plant PME generally demethylesterifies pectin in a blockwise (progressive) manner, creating long sequences of non-methylesterified GalA units. Pectin samples demethylesterified by plant PME therefore exhibited different DMs with blockwise patterns of methylesterification.

Lastly, recombinant *Aspergillus aculeatus* PME was purified from a commercial liquid preparation as described by Duvetter et al. (2005) and used to demethylesterify commercial

pectin as detailed by Fraeye et al. (2009). To 25 mL of pectin solution (0.8% w/w high DM citrus pectin, DM ~ 94%), 15 U of fungal PME was added and the mixture was incubated for desired periods at 30 °C. The PME was thereafter inactivated in a heat shock step of 4 min at 85 °C before the samples were rapidly cooled. All the demethylesterified samples were transferred into dialysis tubes (12-14 kDa molecular weight cut-off, (MWCO)) and dialysed against demineralised water before lyophilization. According to Willats et al. (2001), the action of fungal PME is generally regarded as rather random (or multiple chain mechanism), resulting in the demethylesterification of single GalA residues. Pectin samples demethylesterified by fungal PME therefore exhibited different DMs with rather random patterns of methylesterification.

2.2. Protein enrichment of pectin samples

Commercial citrus pectin (DM ~ 94%) and plant PME demethylesterified pectins of DM 60.8, 74.3 and 14.8% were dissolved in demineralised water, after which the pH was adjusted to 6. The samples were transferred into Spectra/Por® dialysis tubing (3.5 kDa, MWCO) and dialysed against demineralised water for 48 h, after which they were freeze dried. To each sample, either 10, 20 or 30% (w/w) bovine serum albumin, BSA, (≥ 96%, Sigma-Aldrich, USA) was added and the mixtures were dissolved before lyophilization.

2.3. Preparation of plant-based materials (real systems)

Various plant tissues including broccoli, carrot, sugar beetroot, tomato, mango and apple were selected and subjected to different processing and storage conditions (see sections 2.3.1- 2.3.7) in order to create a wide range of pectin DMs. The processing and storage conditions performed in this study were based on previous in house experience. Cell wall material was subsequently isolated as alcohol insoluble residue (AIR) (see section 2.4).

2.3.1. Broccoli tissue

Broccoli (*Brassica oleracea* L. cultivar italica) from a local shop were stored at 4 °C for a maximum period of five days before use. Small broccoli stems were collected and either not treated or (pre)processed by thermal- or high-pressure treatments in order to stimulate the endogenous PME activity and allow demethylesterification of pectin in broccoli tissue. Sample preparation and treatments were performed according to Christiaens et al. (2011). The treated samples were frozen and ground in a Grindomix mixer (Retsch GM 200, Germany) before extraction of the cell wall material.

2.3.2. Broccoli purée

Broccoli purée was prepared according to the method of Christiaens et al. (2012a). Broccoli (*Brassica oleracea* L. cultivar italica) florets were either not pretreated or subjected to low- or high-temperature blanching (40 min, 60 °C and 5 min, 95 °C respectively). Depending on the intensity of the thermal treatment, PME activity was either activated or inactivated. Broccoli purée was subsequently created by blending or blending followed by high-pressure homogenisation at 100 bar. Selected broccoli purées were further subjected to a cooking step of 20 min at 120 °C.

2.3.3. Carrot purée

Fresh carrots (*Daucus carota* cultivar Nerac) stored for a maximum of two days at 4 °C were peeled and cut into one cm^3 pieces. The pieces were either pretreated at low or high temperature (40 min, 60 °C and 5 min, 95 °C respectively) or not pretreated to result in different PME activity. Purée was prepared from the (pretreated) samples by blending or blending followed by high-pressure homogenisation at 100 bar as described by Christiaens et al. (2012b).

2.3.4. Tomato purée

Process tomatoes (*Lycopersicum esculentum* cultivar Patrona) at a red-ripe stage were purchased from Spain and used to create different tomato tissue particle suspensions as described in the method of Christiaens et al. (2012c). The tomatoes were either high-pressure pretreated (10 min, 25 °C and 550 MPa) to selectively maintain PME activity while inactivating polygalacturonase activity, blanched (8 min, 95 °C) to inactivate PME and polygalacturonase or maintained as raw sample. Purée was prepared from the (pretreated) tomatoes by blending or blending followed by high-pressure homogenisation at 100 bar.

2.3.5. Sugar beet tissue

Sugar beetroots (*Beta vulgaris* cultivar Sabrina) were freshly harvested from a plant in Belgium. Cylinders (diameter 12 mm and height 10 mm) were excised from the sugar beetroot tissue before being subjected to either thermal or high-pressure processing. On the one hand, sugar beet cylinders were vacuum sealed in a polyethylene bag and thermally treated in a water bath for 15 min at 80 °C. On the other hand, 10 sugar beet cylinders were placed in metal tubes (length 110 mm, internal diameter 13 mm and thickness 1 mm) and subsequently filled with demineralised water. The tubes were then heated in an oil bath (15 min, 100 °C; 45 min, 100 °C and 6 h, 100 °C) and thereafter cooled in an ice bath, frozen with liquid N_2 and stored at -40 °C. High-pressure processing was performed in a single-vessel, laboratory-scale high-pressure equipment (Engineered Pressure Systems International, EPSI, Temse, Belgium). A mixture of propylene and glycol (60% Dowcal, Dow Chemical Co., Horgen, Switzerland) was used as the pressure-transmitting medium. To thermostat the system, an external heat exchanger using 58% ethylene glycol solution (Cryostat Haake N8-KT 50W, Karlsruhe, Germany) was used. Sugar beet

samples were vacuum packed in double-film polyethylene bags and high-pressure treated for either 30 min at 25 °C and 400 MPa or 30 min at 60 °C and 400 MPa. After the treatments, samples were cooled to room temperature in an ice bath, frozen with liquid N_2 and stored at -40 °C. For further use, the frozen sugar beet cylinders were ground in a Grindomix mixer (Retsch GM 200, Germany) before isolation of the cell wall material.

2.3.6. Apple tissue

Apples (*Malus domestica* Borkh cultivar Jonagold) were harvested at a commercial optimal picking date and stored under different conditions. The apples were either stored at 1 °C or 4 °C in four differently controlled atmosphere conditions (1 kPa O_2 and 3 kPa CO_2; 1 kPa O_2 and 10 kPa CO_2; 3 kPa O_2 and 3 kPa CO_2; 3 kPa O_2 and 10 kPa CO_2). After a storage period of six months in controlled atmosphere, the apples were stored at shelf conditions (20.8 kPa O_2, 0.03 kPa CO_2 and 18 °C) for a maximum of 14 days. To isolate cell wall material, apples were drawn at an interval of 7 days during shelf storage conditions and a 3 months interval during controlled atmosphere storage. After peeling, the apple flesh was cut into one cm^3 cubes, the cubes were frozen and ground in a Grindomix mixer (Retsch GM 200, Germany) before extraction of the cell wall material.

2.3.7. Mango tissue

Ngowe mangoes (*Mangifera indica*) and Totapuri mangoes at full maturity were obtained from Kenya and India respectively. The mangoes were either coded raw (not ripened), or ripened at room temperature (25 °C) for 2-3 days (ripe sample) or 4-5 days (overripe samples). The samples were then peeled and sliced before the processing treatments were performed. Samples were either not treated or thermally treated. Untreated samples were prepared as mango pieces cut into

243 one cm^3 cubes and frozen with liquid N_2. For thermal treatment, 30 mm slices were vacuum
244 sealed and heat treated in a water bath (8 min, 95 °C). The heat treated samples were
245 immediately cooled in an ice water bath and frozen with liquid N_2. For further use in cell wall
246 material isolation, the frozen mango cubes or slices were first ground in a Grindomix mixer
247 (Retsch GM 200, Germany).

248 **2.4. Isolation of cell wall material**

249 Plant cell wall material from differently treated plant tissues (see section 2.3) was isolated as
250 alcohol insoluble residue (AIR) using the method described by McFeeters and Armstrong
251 (1984). Approximately 30 g of sample was homogenised in 192 mL of 95% ethanol using a
252 mixer (Büchi mixer B-400, Flawil, Switzerland). The resulting suspension was filtered
253 (Machery-Nagel MN615 ϕ 90 mm) and the residue obtained was rehomogenised in 96 mL of
254 95% ethanol. After a second filtration step, the resulting residue was homogenised in 96 mL of
255 acetone. The final residue after the last filtration step was dried overnight at 40 °C to obtain AIR,
256 which was then stored over P_2O_5 in a desiccator until further use.

257 **2.5. Extraction of water soluble pectins**

258 Water soluble pectins were extracted from AIR using the following procedure. To exactly 0.25 g
259 of AIR, 45 mL of boiling demineralised water was added and the suspension was boiled for 5
260 min with constant stirring (Sila, Smout, Elliot, Van Loey, & Hendrickx, 2006). After cooling, the
261 mixture was filtered (Machery-Nagel MN615 ϕ 90 mm) and the filtrate obtained (containing the
262 water soluble pectins, WSP) was adjusted to 50 mL with demineralised water.

263 **2.6. Determination of degree of methylesterification**

The DM of the pectin in the samples was determined using both colorimetric and FT-IR spectroscopy methods.

2.6.1. Colorimetric method

The DM of pectin in the different samples was determined as the ratio of the molar amounts of methylesters to the molar amounts of GalA and expressed as a percentage. GalA content was determined by first hydrolyzing pectin material (in duplicate) with concentrated sulfuric acid based on the method described by Ahmed and Labavitch (1977). Hydrolysed samples were subsequently diluted with demineralised water and the GalA concentration was analysed according to the spectrometric method of Blumenkrantz and Asboe-Hansen (1973). The analysis was performed in triplicate. Samples for methanol quantification were hydrolysed (in duplicate) under alkaline conditions to release methanol as described in the method of Ng and Waldron (1997) with minor adaptations. The hydrolysate was diluted with phosphate buffer (pH 7.5) and the released methanol was enzymatically oxidized to formaldehyde. The methanol content was subsequently quantified based on the formaldehyde formed as described in the colorimetric method of Klavons and Bennett (1986). The samples were analysed in triplicate.

2.6.2. Fourier Transform Infra-Red (FT-IR) method

Approximately 10 mg of calibration sample, AIR or WSP was dissolved in demineralised water, after which the pH was adjusted to 6 with NaOH (0.1 N or 0.01 N). In case of liquid samples, the filtrate was directly adjusted to the desired pH. As the pKa of polygalacturonic acid is 3.38, adjusting the pH to 6 guarantees total ionization of the carboxylic groups (Manrique & Lajolo, 2002). The samples were transferred into Spectra/Por® dialysis tubing (3.5 kDa, MWCO) and dialysed against demineralised water for 48 h, after which the dialysed solutions were lyophilised

and stored over P_2O_5. For DM determination, a small sample was firmly compacted to expel entrapped air and ensure smooth surfaces, placed on the sample holder of the FT-IR (Shimadzu FTIR-8400S, Japan) and the transmittance measured at wave numbers from 4000 cm^{-1} to 400 cm^{-1} at resolution 4 cm^{-1}. To reduce the noise to signal ratio, 100 scans were run per sample and integrated to obtain spectra mean values. The spectra were converted into absorbance mode before base line correction, after which further spectra processing was performed. The spectra were either not deconvoluted or deconvoluted to correct for the interference of protein present in the samples. After spectra preprocessing, the absorption intensity of the bands situated around 1740 cm^{-1} (due to ester carbonyl group (C=O) stretching) and 1630-1600 cm^{-1} (due to carboxylate group (COO^-) stretching) (Szymanska-Chargot & Zdunek, 2013) were used to predict the DM.

3. Results and discussion

3.1 Analysis of FT-IR spectra: peak deconvolution using model systems

Before addressing the FT-IR calibration curves, the option of peak deconvolution as a tool to correct for any interferences due to the presence of proteins in the pectin samples will be discussed here. Presented in Figure 1 are the spectroscopic profiles for the bands in the region relevant to this study (1800 to 1500 cm^{-1}). The pectin sample spectra showed bands at around 1740 cm^{-1} and between 1630-1600 cm^{-1}, with some samples spectra also exhibiting bands at around 1650 cm^{-1} and 1543 cm^{-1}. The spectrum of pure BSA as presented in Figure 1(A) and (B) exhibited two absorption peaks at 1643 and 1530 cm^{-1}. The bands of particular interest for DM determination appeared at 1630-1600 cm^{-1}, due to stretching of the carboxylic group (COO^-) and

around 1740 cm^{-1}, due to stretching of the C=O bond of the ester carbonyl group (Manrique & Lajolo, 2002). Furthermore, based on the observations of McCann et al. (1997), the peaks at around 1650 and 1543 cm^{-1} were assigned to protein amide stretches. Figure 1(A) represents the absorbance spectra of a high DM pectin (DM 95.4%) with different amounts of added protein while Figure 1(B) shows the absorption spectra of low DM pectin (DM 14.8%) with different amounts of added protein. From low to high DM, an increase in intensity of the band around 1740 cm^{-1} and a simultaneous decrease in intensity of the peak situated at 1630-1600 cm^{-1} was observed as previously reported by Gnanasambandam and Proctor (2000), and Szymanska-Chargot and Zdunek (2013). In addition, changes in spectra shape and peak shifts (particularly the peak at 1600 cm^{-1}) to higher wave numbers were observed with increasing DM and protein content. According to Szymanska-Chargot and Zdunek (2013), in the case of plant cell walls, a shift of bands can be attributed to polysaccharides being bonded with each other or complexing of pectic carboxylate groups with calcium ions (Chatjigakis et al., 1998). Furthermore, this peak shift could also result from small differences in structure and constitution of a molecule in addition to differences in the type and content of pectin side chains (Gnanasambandam & Proctor, 2000).

According to Chatjigakis et al. (1998), presence of protein compounds and water could affect DM determination by contributing to the intensity of the band at 1630 cm^{-1} used in DM determination. To eliminate the interference of water, the samples were extensively freeze dried and stored over P_2O_5 while peak deconvolution was performed as shown in Figure 1(C) in order to correct for protein interference. In Figure 1 (A) and (B), the increase in amount of added protein was reflected in the increased intensities of the peaks at 1653 and 1541 cm^{-1} which is in agreement with the findings of Sene et al. (1994) that the presence of proteins gives rise to two

strong bands arising from amide linkages (around 1650 and 1550 cm^{-1}), often present in a ratio of about 2:1. Peak deconvolution (Gaussian peak type) resulted in individual peaks centered at approximately 1650 cm^{-1} and 1540 cm^{-1} for protein, 1630-1602 cm^{-1} for the carboxylic group and around 1740 cm^{-1} for the carbonyl ester group. The need for peak deconvolution (peak splitting) was evaluated by spiking pectins of low to high DM with BSA in amounts representative of the natural protein content in some of the selected fruits and vegetables. The protein contents on dry matter basis range from as low as 1% in apple and 1-4% in mango to 34 % in broccoli (Hassan, Al-sheraji, & Ismail.A, 2013; Marino et al., 2010). Intermediate protein contents have been reported for sugar beetroot (3-7%), carrots (6-13%), and tomatoes (15-21%) (Goby & Gidenne, 2008; Walker, Jenkins, & Klopfenstein, 2011; Gupta, Kawatra, & Sehgal, 2011; Marino et al., 2010).

Table 1 shows the FT-IR determined DM values of selected calibration samples with no added protein, the amounts of added protein, as well as the DM values obtained for the different calibration samples with and without deconvolution of the FT-IR spectra collected for the protein enriched samples. The DM values of samples with no added protein were determined using the standard calibration line (see further, Figure 2A). After addition of 10-30% BSA protein, the DM values of the samples were determined without peak deconvolution using the linear fit in Figure 2(A) while DMs with spectra deconvolution were obtained using the calibration line in Figure 2(B). It was observed that at low DM (DM 14.8%), the determined DM values with and without peak splitting did not show significant differences at all levels of added protein. This implies that at low DM, irrespective of protein content, accurate DM determination can be made even without peak deconvolution. For the intermediate DMs (DM 60.8% and 74.3%) tested, the importance of peak splitting became evident when the amount of protein was increased to 30%.

354 Predicting the DM of these pectin samples without peak splitting resulted in a significant
355 underestimation of the DM while deconvolution of the spectra gave a high accuracy of the
356 determined value. At the highest DM (DM 95.4%) investigated, underestimation is already
357 evident at 20% protein added, with the sample exhibiting up to 40% determination error as the
358 protein content is increased to 30%. From these results, it could be concluded that whereas peak
359 deconvolution is not relevant at low DM, as the DM increases, particularly in combination with
360 high protein content, it becomes vital to perform spectra deconvolution before DM analysis.

361 **3.2 FT-IR calibration curves**

362 Standard calibration curves were obtained by plotting the colorimetrically determined DMs for
363 the demethylesterified pectin samples or theoretically determined DMs for the mixtures of PGA
364 and citrus pectin against the FT-IR results. A linear regression analysis was then performed to
365 obtain the relationship between DMs determined for the two sets of calibration samples and the
366 FT-IR spectral band intensity ratio as described by Gnanasambandam et al. (2000). On the one
367 hand a linear regression analysis was performed to obtain the relationship between the
368 colorimetrically determined DM values of a series of demethylesterified pectins with different
369 patterns of methylesterification and the FT-IR spectral band intensity ratio. On the other hand,
370 the relationship between theoretically determined DM values of mixtures of PGA and
371 commercial citrus pectin and the FT-IR spectral band intensity ratio was established. High
372 squared correlation coefficients (R^2) were obtained for the plot of colorimetrically determined
373 DMs against FT-IR results as well as for the plot of theoretically determined DMs against DM
374 values obtained by FT-IR (results not shown separately). Subsequently, in order to create an
375 overall standard curve, all colorimetrically and theoretically determined DM values were plotted
376 together against the FT-IR results in a single plot. Figure 2(A) shows the overall calibration

curve of the DMs determined for the two sets of calibration samples against the ratio of peak intensity at 1740 cm^{-1} to the sum of peak intensities at 1740 cm^{-1} and 1600 cm^{-1} without peak deconvolution. In Figure 2(B), the FT-IR spectra were deconvoluted before the ratios were computed. Using mixtures of PGA and high DM commercial citrus pectin, DM values between 0 and 94% were obtained. The chemically and enzymatically demethylesterified pectin series yielded DMs between 10 and 85%. These pectins exhibited different DMs in addition to different patterns of methylesterification (Ngouemazong et al., 2011). Linear regression analysis showed good correlation in both cases. A slope closer to 100 was obtained for the plot with DM values of deconvoluted spectra compared to the slope in the case without spectra deconvolution. The high correlation obtained between colorimetrically or theoretically determined DMs and the FT-IR peak intensity ratio not only provides a good calibration line but it also indicates that FT-IR can be used to predict DM of pure pectin irrespective of the pectin nanostructure in terms of demethylesterification.

3.3 Determination of degree of methyl esterification of AIR

Using the calibration lines in Figure 2, the DM of AIR of real systems (different fruit and vegetable samples) was determined. Presented in Figure 3(A) and (B) are plots of the colorimetrically determined DM of AIR against the DM determined using FT-IR without and with spectra deconvolution respectively. Samples represent a broad range of DM and protein content as they were obtained from various fruits and vegetables that were differently matured, stored or processed. Linear regression analysis in both cases showed a slope close to 1 indicating a good linear correlation between the colorimetrically determined DM and the FT-IR determined DM. In addition, high squared correlation coefficients were obtained in both cases. Peak

deconvolution did not seem to improve the correlation, in fact the slope of the linear fit deviated more from 1 and a slight drop in the correlation coefficient was observed.

Looking at specific types of samples, the DM of AIR from tomato purées was well determined with and without deconvolution of the FT-IR spectra. Similar observations were made for sugar beetroot, carrot, apple and mango samples ranging from intermediate to high DM. In the case of broccoli, although the correlation between colorimetric and FT-IR values was lower compared to other fruits and vegetables, the DM of broccoli AIR samples (ranging from 35 to 58%) seemed to be well determined without peak deconvolution. This rather good determination was unexpected since broccoli has been reported to contain high amounts of protein, and as stated earlier, protein interferes with DM determinations using FT-IR. Deconvolution of broccoli spectra resulted in a shift of the FT-IR determined DM values to significantly higher values. The lower correlation between the colorimetrically determined DM and the DM determined by FT-IR in the case of broccoli might however be related to problems encountered in the colorimetric estimation of the DM. During AIR extraction, some of the green colour of broccoli was retained by the AIR and could have interfered with methanol and GalA quantification. Other compounds, including neutral sugars (Anthon & Barrett, 2008) and phenolics naturally present in the sample could also have interfered with the colorimetric measurements. In general, DM of AIR samples of various fruits and vegetables at different maturity stages, stored at various conditions or subjected to different (pre)processing techniques can be accurately determined using FT-IR with or without peak deconvolution, particularly for low protein samples.

3.4 Determination of degree of methyl esterification of WSP

The DM of WSP of the samples was determined with and without spectra deconvolution (Figure 4 A and B respectively) using the calibration lines of Figure 2. A slight improvement in the squared correlation coefficient of the linear correlation between the colorimetrically determined DMs and the FT-IR determined DM values was observed with peak splitting. However, the slope of the linear fit was closer to 1 in the case of non-deconvoluted spectra compared to deconvoluted spectra. By excluding the lower DMs and predicting the medium to high DMs, to eliminate the cluster behavior, a significant improvement of the slope was observed with peak deconvolution. The slope value increased from 0.731 without peak deconvolution to 0.915 with peak deconvolution (results not shown). It was also evident that broccoli samples exhibited the largest deviation from the linear fit. By omitting the broccoli samples and predicting the DM of pectin of the remaining samples, higher squared correlation coefficients between colorimetrically determined DM values and the FT-IR values were obtained (in both case, with and without peak splitting, R^2= 0.943 was obtained). Predicting broccoli DM separately resulted in low squared correlation coefficients of 0.512 and 0.300 with and without peak deconvolution respectively (results not presented). The slopes associated with these coefficients were 0.867 and 0.889 respectively. Although spectra deconvolution seemed to improve the squared correlation coefficient, the correlation was still low. As in the case of AIR, colorimetric measurements could have been affected by the high protein content of broccoli. In fact, Anthon and Barrett (2008) suggested that high levels of protein interfere in the *m*-hydroxydiphenyl assay for GalA. As the FTIR deconvolution hypothesis was only tested in model systems, extending this to real systems could have resulted in deviations as the protein (BSA) used does not accurately represent the protein in real systems and its interaction with other components is probably different from the interactions of proteins in real samples. In general, DM of WSP samples of various fruits and

vegetables at different maturity stages, stored at various conditions or subjected to different (pre)processing techniques can be accurately determined using FT-IR without peak deconvolution. Incase protein-rich samples are investigated, peak splitting is vital for good determination.

4. Conclusion

FT-IR provides considerable advantages compared to colorimetry as a method for determining DM of pectin samples because of its simplicity, speed, high sensitivity and practicability. This study provides more evidence for use of FT-IR spectroscopy as a reliable method for routine analysis of DM of AIR and WSP of different fruits and vegetable tissues irrespective of maturity stage, storage condition, and processing treatment. A single standard calibration curve based on pectins demethylesterified through different methods and mixtures of PGA and citrus pectin is sufficient for this purpose. FT-IR can thus be used for fast screening of the DM during production of pectin or pectin research. When protein rich samples are investigated, peak deconvolution should be performed to improve the determination accuracy. In general, owing to the fact that most fruits and vegetables contain low amounts of protein, FT-IR without peak deconvolution is a reliable method for DM determination.

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584

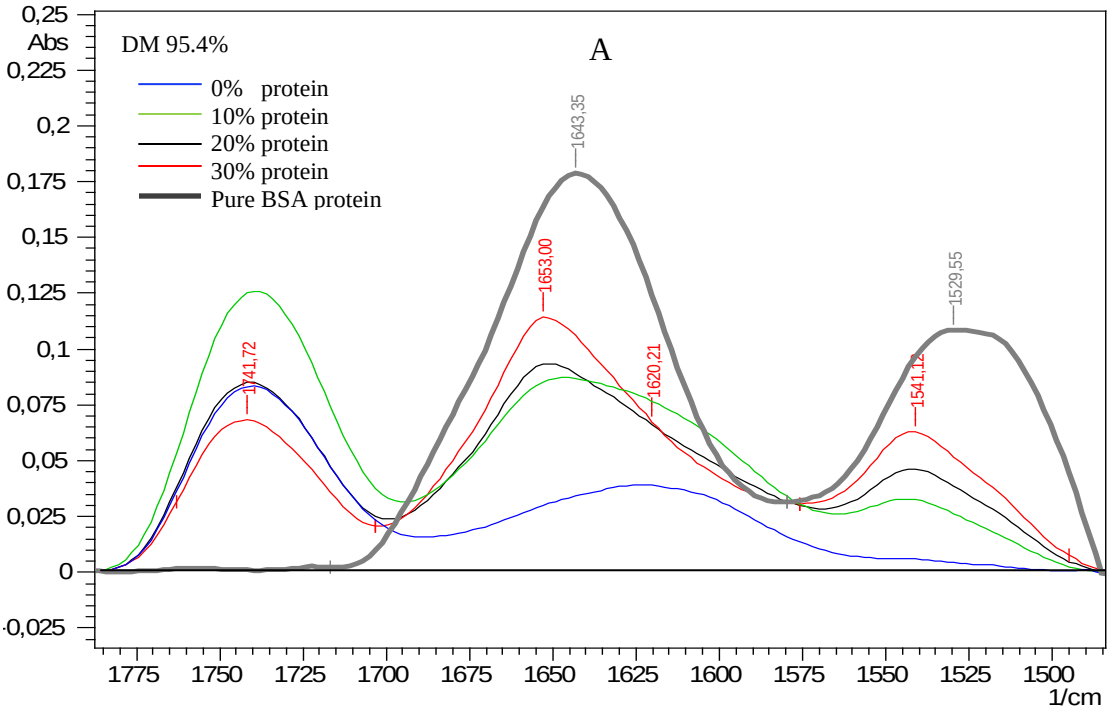
585 **Figure captions**

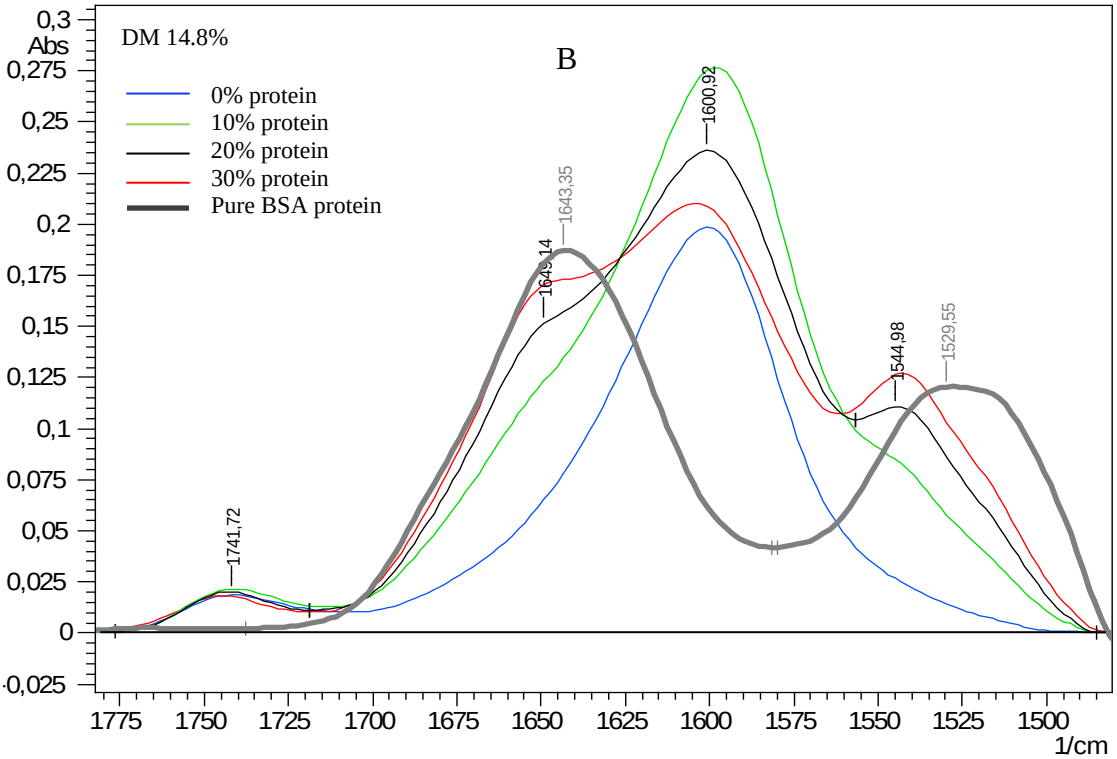
586 **Figure 1.** (A) FT-IR spectra of high DM citrus pectin (95.4%) spiked with 0-30% BSA protein,
587 without spectra deconvolution. (B) FT-IR spectra of low DM citrus pectin (14.8%) spiked with
588 0-30% BSA protein, without spectra deconvolution. In both Figure (A) and (B), a spectrum for
589 pure BSA is shown. (C) Deconvoluted FT-IR spectra of a low DM citrus pectin (DM 14.8%
590 spiked with 30% BSA).

591 **Figure 2.** Calibration curves for DM determination using FT-IR, with (◆) representing the
592 colorimetrically determined DM of demethylesterified pectin series and (▲) representing the
593 theoretically calculated DMs of mixtures of PGA and commercial citrus pectin, as a function of
594 the ratio of peak intensity at 1740 cm^{-1} to the sum of peak intensities at 1740 cm^{-1} and 1630-1600
595 cm^{-1}. The intensity at 1630-1600 cm^{-1} is represented by I (1600). (A) Calibration curve of the
596 theoretically and colorimetrically determined DMs against FT-IR values obtained from non-
597 deconvoluted spectra. (B) Calibration curve of the theoretically and colorimetrically determined
598 DMs against values determined using FT-IR with spectra deconvolution. The equations and
599 squared correlation coefficients (R^2) of the linear fit are displayed on the graphs.

600 **Figure 3.** (A) The DM of AIR determined by the colorimetric method against the DM of AIR
601 determined using FT-IR spectroscopy without peak deconvolution. (B) The DM of AIR
602 determined colorimetrically against the DM of AIR determined with FT-IR spectroscopy with
603 peak deconvolution. The equations and squared correlation coefficients (R^2) of the linear fit are
604 displayed on the graphs.

Figure 4. (A) The DM of WSP determined by the colorimetric method against the DM of AIR determined with FT-IR spectroscopy without peak deconvolution. (B) The DM of WSP determined colorimetrically against the DM determined with FT-IR after peak deconvolution. The equations and squared correlation coefficients (R^2) of the linear fit are displayed on the graphs.


DM 95.4%
A
0% protein
10% protein
20% protein
30% protein
Pure BSA protein
Abs
1/cm
1643,35
1653,00
1620,21
1529,55
1741,72


DM 14.8%
B
0% protein
10% protein
20% protein
30% protein
Pure BSA protein
Abs
1/cm
1600,92
1643,35
1649,14
1544,98
1529,55
1741,72

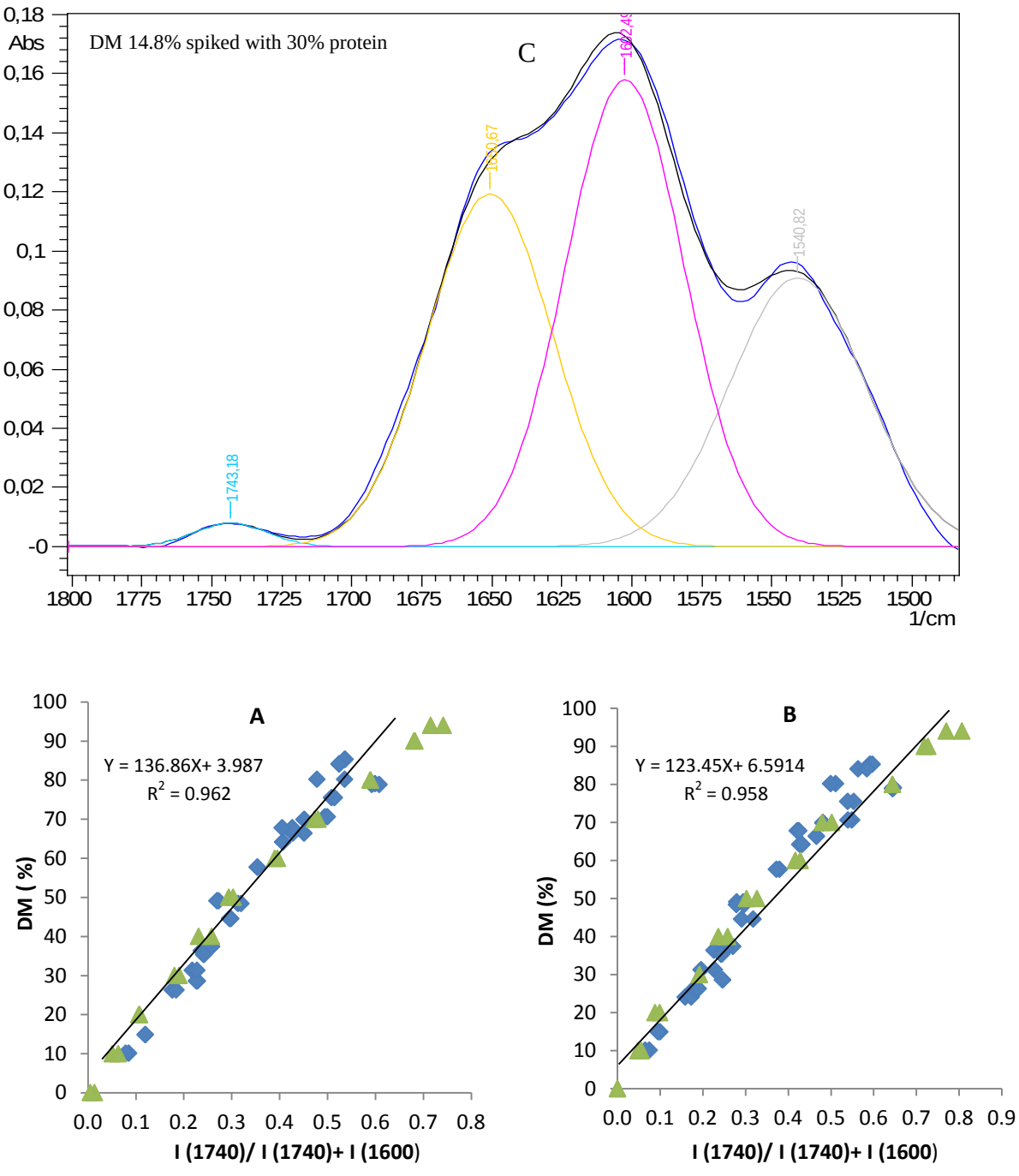

DM 14.8% spiked with 30% protein
C
Abs
1743,18
1620,67
1602,49
1540,82
1/cm
A
Y = 136.86X+ 3.987
R2 = 0.962
DM (%)
I (1740)/ I (1740)+ I (1600)
B
Y = 123.45X+ 6.5914
R2 = 0.958
DM (%)
I (1740)/ I (1740)+ I (1600)

Figure 2.

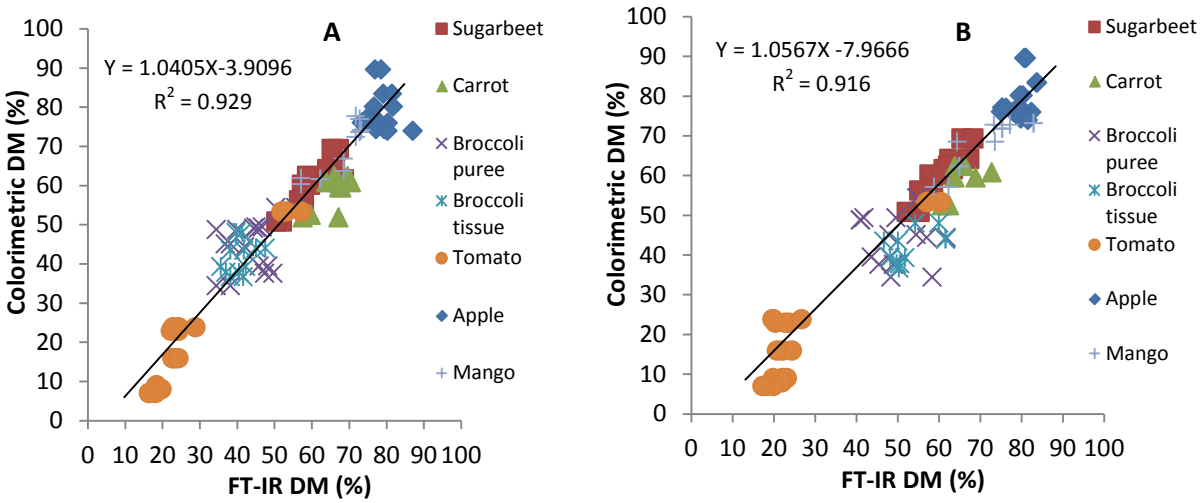


Figure 3.

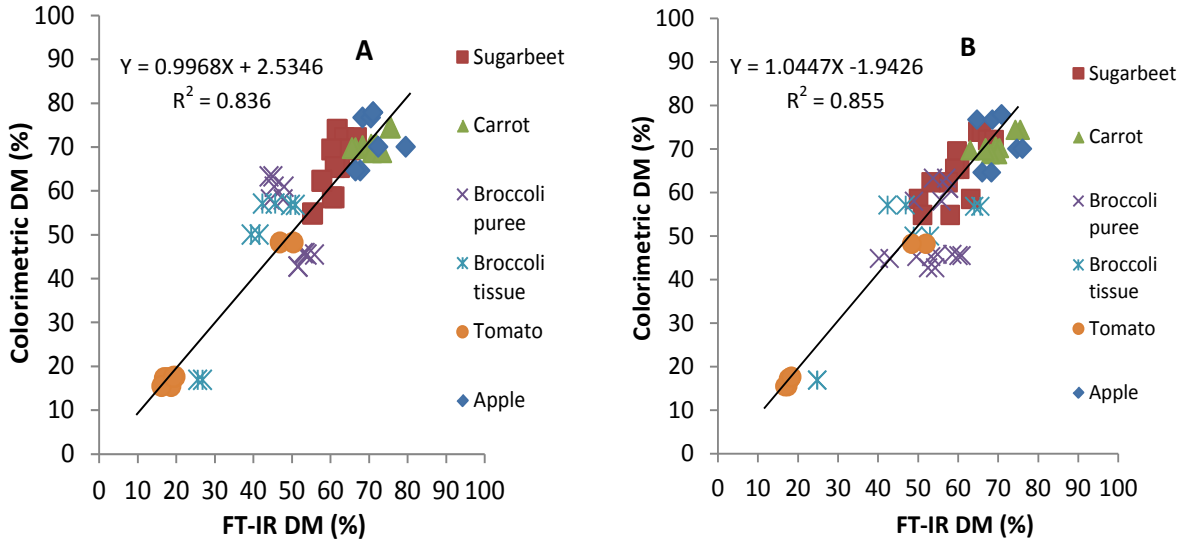


Figure 4.

DM (%) (no protein added)	BSA (%)	DM (%)	
		Without deconvolution of FT-IR spectra	With deconvolution of FT-IR spectra
14.8 ± 1.12	10	14.8 ± 0.62	14,8 ± 0.91
	20	15.3 ± 0.51	15,7 ± 2.60
	30	15.1 ± 0.12	15.2 ± 0.05
60.8 ± 2.08	10	64.0 ± 1.11	63.8 ± 0.30
	20	60.5 ± 0.73	62.1 ± 2.89
	30	44.1 ± 2.49	59.1 ± 2.08
74.3 ± 0.33	10	77.4 ± 0.46	78.6 ± 0.91
	20	73.5 ± 1.54	75.4 ± 1.10
	30	54.6 ± 1.20	72.2 ± 0.13
95.4 ±1.87	10	89.3 ± 0.02	97.3 ± 3.17
	20	72.9 ± 0.75	95.5 ± 2.84
	30	57.2 ± 0.13	92.4 ± 2.40

Table 1. A presentation of the FT-IR determination of DM of pectin of selected calibration samples without added protein and with different levels of added protein. The results of DM of pectin predicted with and without spectra deconvolution are shown with the associated standard deviation (± SD) of duplicate values from the average.